Osteocyte Signalling in Response to Mechanical Loading after Physical Cellular Damage and Hyperglycemia

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy in Biomedical Engineering
Institute of Biomaterial and Biomedical Engineering
University of Toronto

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Abstract

Osteocytes are cells that reside in the mineralized bone matrix. They are the cells
that sense mechanical loading on the bone; and respond by directing biochemical signals
to osteoclasts (for bone resorption) and osteoblast (for bone formation) to remodel the
bone. However, it is unknown how mechanical forces affect signaling of osteocytes that
are affected by physical damage and pathological chemical environment.

In the bone, mechanical loading causes micro-cracks, which cause physical
damage to osteocytes and initiate bone remodeling. However, the role of subsequent
mechanical loading on osteocyte is unclear. In this study, we have developed an in vitro
cell model to study the impact of mechanical loading on osteocytes with physical
damage. This model re-created the spatial distribution of osteocyte and signals expression
around micro-cracks as in vivo. The results showed that 1) subcellular physical damage
upregulates remodeling signals in osteocytes at 24 hr after damage, 2) mechanical
loading substantially upregulates these signals for remodeling in osteocytes with physical damage.

Hydraulic pressure and fluid flow shear stress are two concurrent mechanical stimuli on osteocytes, since the fluid shear is driven by the pressure gradient in the interstitial fluid in bone. A new mechanical loading system was developed to apply the concurrent mechanical forces. The loading system was able to safely apply physiological loading magnitudes to cells cultured on glass slides. It was found that concurrent pressure and shear stress had an additive effect on the reduction of osteocyte apoptosis.

High glucose concentration in diabetic patients has resulted in retarded bone accumulation, bone loss, and an elevated risk of bone fracture. Osteocyte sensitivity to mechanical loading could be a factor in this pathology. The hypothesis is that hyperglycemia suppresses the beneficial effects of fluid flow shear stress on osteocytes. We found that high glucose level abolished mechanical-loading-induced changes in osteocyte signals to regulate bone remodeling, while osmotic control medium having the same elevated osmolarity did not have significant effects. Elevated glucose levels in diabetic patients could have direct effects on osteocytes, adversely influencing osteocyte response to mechanical loading, consequently the bone remodeling process.
Acknowledgments

These past five years truly had been a learning experience, in terms of research skills and personal growth.

I would like to thank my supervisor, Dr. Lidan You, for giving me the opportunity to study in such a great lab. Dr. You has provided support and guidance throughout my graduate studies. Lidan, thank you for giving me the intellectual freedom to investigate a field of study that is a passion of mine. Throughout this investigation, your insightful questions and advice helped me to stay on the right track. Last but not least, your encouragement helped to overcome the toughest of times. I am very excited about the research direction of your lab, and I am excited to read about your new studies in the future.

I also thank my committee members Dr. Craig Simmons and Dr. Benjamin Alman. Dr. Simmons, thank you for sharing your and insights. Many times, you have revealed new perspectives to the implications my data. This had, in turn, motivated me to look deeper and finding more interesting results. Dr. Alman, thank you for sharing your vast knowledge of bone biology and clinical experience. You helped me in thinking about the relation of my experiments in a larger perspective.

To my lab-mates, first are Esther, Yan, Saja, and Wing-Yee. You guys helped me getting started in the lab. I would not have learned the protocols half as quickly without you. Kevin, thank you for your knowledge as the resident mechanical/material engineer of the lab. Also your company is much appreciated throughout the years. Jeff, from Stanford and Davies Labs and the unofficial member of our lab, thank you for sharing
your cell biology knowledge with me. To the newest grad students, Vivian and Madeleine, you have kept the morale high for the lab as well as for me.

To the undergraduate students that I have the pleasure of working with, thank you for all the hard work during your time in the lab. I know each one of you have made significant contributions in your respective projects.

Last but not least, to my family members, who have provided sustained and full support for my studies. A special thank goes to my wife, despite having to tolerate my long hours at the lab, has created a home for us where I could truly relax and recharge. Thank you.
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Overview of contributions

The works presented in this thesis were made possible by many colleagues that worked with me during my years as a PhD candidate. This section outlines their contribution to the work presented in this thesis, as well as my contributions.

Chapter 4 describes the study of designing an in vitro cell damage model to mimic the cell damage that osteocytes sustain in bone micro-cracks. The system was a novel design. I defined the functional requirement of the system, mainly: capability to cause sub-cellular damage for cells seeded on glass slide. I made engineering CAD drawings of the system, selected parts, machined custom parts not commercially available, and assembled the system. I tested and refined the system to be able to apply sub-cellular damage to cultured MLO-Y4 cells. I used Trypan Blue, LIVE/DEAD, caspase 3/7, and VEGF staining to validate the damage profile. This system was used to investigate how physical damage affect osteocyte mechanotransduction, and their ability to signal for bone remodelling. Xiaoqing Zhang and Michael Wu, who were both summer students in 2013, assisted me in setting up of the parallel flow chamber and the loading system. They also helped me in collecting mRNA and ELISA measurements. Michael also helped me with LIVE/DEAD staining and quantification. I performed the data analysis for all the experiments. I used the conditioned media from the cell damage and mechanical loading experiments to induce osteoclast formation. I optimized the duration of conditioned media exposure and the concentration of baseline RANKL during the differentiation experiments. Then I performed TRAP staining and quantification to measure mature osteoclasts. I wrote a full-length manuscript to present the results from this study. The
manuscript had been submitted to the Journal of Biomechanics, and is under revision at the time of writing of this thesis.

Chapter 5 describes the study of engineering a system that is capable of applying two modes of oscillatory loading, hydraulic pressure and fluid shear stress, concurrently to cultured osteocytes. I designed a hydraulically closed loop load-application system with independent control for the pressure and shear stress loading magnitude and frequency, with overpressure protection and media reservoir. I validated the loading profile through MatLab Simulink simulation. Using SolidWorks, I made chamber designs for each iteration of the prototype. The final working prototype was able to ensure accuracy of shear stress and pressure loading to be within 5% nominal. I made the part selection and optimization for the loading circuit and the loading chamber. Xiaoqing Zhang, who was a summer student in 2013, assisted me in testing the loading circuit function. Franklin Xi Sun, who was a summer student in 2014, assisted me in prototyping and iterative optimization of the loading chamber. Lily Lau, who was an undergraduate thesis student in 2014-2015, helped me in system integration and testing of mechanical function and cell compatibility. Tong Xu, who was a summer student in 2015, helped me with data collection for apoptosis assay and immunofluorescent staining of the cytoskeleton. I completed analysis for all of the data from this study. I have written a manuscript presenting the results from this study. This manuscript is under preparation, and will be submitted as a short technical report in a peer reviewed publication.

In Chapter 6, I describe the study on the effect of hyperglycemia on osteocyte mechanotransduction. This was a collaborative project with Dr. Liyun Wang at the University of Delaware. I contributed in vitro results from mechanical loading
experiments on cultured osteocytes. I performed experiments in which cultured osteocytes were subjected to fluid shear stress in hyperglycemic conditions. Hashem Al-Dujaili, who was a summer student in 2012, assisted me the assembly of parallel flow chambers and loading system, and data collection. The results were published in Bone in 2015.

**Peer-reviewed Journal Publications**

1. **Chao Liu**, Xiaqing Zhang, Michael Wu, Lidan You “Mechanical Regulation of Osteocyte Responses to Physical Damage *in vitro*” Journal of Biomechanics. 2015


4. Chao Wei, Beiyuan Fan, Deyong Chen, **Chao Liu**, Yuanchen Wei, Bo Huo, Lidan You, Junbo Wang and Jian Chen “Osteocyte Culture in Microfluidic Devices” Biomedicalics. 9, 014109 (2015)


6. **Chao Liu**, Yan Zhao, Wing-Yee Cheung, Ronak Gandhi, Liyun Wang, Lidan You “Effects of cyclic hydraulic pressure on osteocytes” Bone. 46 (5), May 2010, pp. 1449-1456

Manuscript in preparation

1. Chao Liu, Frank Xi Sun, Lily Lau, Lidan You “Loading system to apply concurrent oscillatory hydraulic pressure and fluid shear to bone cells in vitro” Journal of Biomechanics. 2015

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Chapter 1

1. Rationale

Bone disorders such as osteoporosis afflict over 2 million Canadians, and contribute to 80% of hip fractures, which costs $10 billion annually in Canada (Lorrain, Paiement et al. 2003). Within a year, 1 in 5 with osteoporotic fracture will die (Lorrain, Paiement et al. 2003). People living with bone disorders become functionally dependent, and many require long-term home care (Lorrain, Paiement et al. 2003). Many bone diseases, including osteoporosis, are caused by the failure of bone remodelling (Manolagas and Jilka 1995), and micro-crack accumulation. Other pathological conditions such as diabetes could also degrade the load-bearing quality of bone (Hamilton, Jamal et al. 2013).

Bone is a living organ that is able to adapt its composition and structure in response to mechanical stimuli (Frost 1990, Aguirre, Plotkin et al. 2004). It was believed that mechanical forces stimulate cells to maintain tissue homeostasis in bone, through the process of bone remodelling.

Hydraulic pressure and fluid flow shear stress are two concurrent mechanical stimuli on osteocytes. They have been shown to be the dominant forces in bone remodelling at the tissue level (Klein-Nulend 1990, Qin, Kaplan et al. 2003). The cellular mechanism of how bone cells sense and respond to these forces still needs to be elucidated. The effects of fluid shear alone (Tan, Bakker et al. 2008, You, Temiyasathit et
al. 2008) and pressure alone (Rubin 1997, Liu, Zhao et al. 2010) on osteocytes have been studied separately. However, the effects of pressure and flow have not been systematically compared and the effect of both at the cellular level is still unclear.

Micro-cracks, which occurs normally in bone, triggers remodelling along the fracture (Kidd, Stephens et al. 2010). This remodelling also requires mechanical loading \textit{in vivo} (Waldorff, Christenson et al. 2010). At the cellular level, micro-cracks could physically damage osteocytes (Hazenberg, Freeley et al. 2006). Due to their abundance in the bone matrix, and their sensitivity to mechanical loading, osteocytes are likely the cells responsible in controlling micro-crack induced remodelling. However the cellular mechanism of micro-crack induced bone remodelling is still unclear. Furthermore, how mechanical loading regulates the responses of osteocytes to micro-cracks in bone is unclear.

Type 2 diabetes patients have a higher risk of fracture, but they do not have reduction in bone density (Schwartz 2013). The mechanism of this symptom is not well understood. It is possible that the heightened glucose concentration in these patients could directly affect osteocyte’s ability to respond to mechanical loading. Since osteocytes play a major role in controlling the bone remodelling process, bone quality would be degraded if the mechanotransduction process were compromised.

Therefore the proposed thesis was focused on using \textit{in vitro} models to investigate how mechanical loading affect osteocyte response to physical damage as in bone micro-cracks; and osteocyte response to high glucose concentration as in pathological conditions such as diabetes.
Chapter 2

2. Background on osteocyte mechanobiology

Bone remodelling is a process occurs in response to mechanical loading and is regulated by osteocytes (Martin 2000). Osteocytes are cells embedded in bone matrix, and are the putative mechanosensor cells in bone. Osteocytes are essential in normal bone remodelling. Osteocyte malfunction (Phillips, Almeida et al. 2008) or absence (Tatsumi, Ishii et al. 2007) in mouse models leads to bone fragility consistent with that observed in aging and osteoporosis. Specifically, while osteocyte ablation increased bone resorption in mice under physiological loading, the same mice were resistant to the disuse-induced bone loss when their hind limbs were subjected to unloading (Tatsumi, Ishii et al. 2007). Upon stimulation by mechanical loading, they release factors to signal bone resorption by osteoclasts or new bone formation by osteoblasts (Henriksen, Neutzsky-Wulff et al. 2009).

2.1. Mechanical stimuli experienced by bone cells

To understand the mechanism by which mechanical forces affect bone cell fate and function *in vivo* requires the knowledge of the mechanical forces experienced by bone cells. In mature bone tissue undergoing remodelling, current hypotheses suggest that interstitial fluid flow is an important link by which tissue level strain is transmitted to
bone cells. The strain in bone tissue in response to mechanical loads induces the pressure gradient within interstitial fluid and induces its movement within the bone pores, from the lacunar-canaliculuar system where osteocytes reside, to the vascular system (Piekarski and Munro 1977, Jacobs, Yellowley et al. 1998, You, Yellowley et al. 2000, Tan, Kuijpers-Jagtman et al. 2006, You, Temiyasathit et al. 2008) (reviewed in (Fritton and Weinbaum 2009)). Interstitial fluid flow within the lacunar-canaliculuar porosity has been shown experimentally through tracer studies (Knothe Tate and Knothe 2000, Knothe Tate, Steck et al. 2000, Mak, Qin et al. 2000, Tami, Schaffler et al. 2003, Gardinier, Townend et al. 2010). Movement of the interstitial fluid within the geometry of lacunar-canaliculuar and haversian systems would create shear stresses on bone cells, in the range of 0 to 20 dynes/cm² (Mi, Basu et al. 2005, Mi, Fritton et al. 2005). According to recent poroelastic model, the level of pressure that is needed to produce this level of shear stress is around 85 kPa (Goulet, Coombe et al. 2009).

Hydraulic pressure at the lacunar-canaliculuar porosity caused by physiological loading was estimated to be 40 times that induced by blood pressure difference (Zhang, Weinbaum et al. 1998); therefore pressure could present a significant stimulus to osteocytes. However, few studies have been done to study the effect of hydraulic pressure on bone cells and its implications in the bone remodelling process. Human cells experience a small change in static hydraulic pressure (SHP) from supine to standing position. This type of loading is estimated to produce 12% of the peak applied uniform axial compressive stress at the lacunar-canaliculuar porosity (Zhang, Weinbaum et al. 1998). Under physiological activities, such as walking, hydraulic compression is cyclic in nature. Oscillatory loading of bone with 0-18 MPa at 1 Hz was calculated to induce
0.27 MPa fluid pressure at the lacunar-canalicular porosity (Zhang, Weinbaum et al. 1998), indicating pressure could be a significant stimulus for bone remodelling. Recent studies found that the hydraulic permeability of the bone tissue was smaller than the previous model assumed, leading to an even higher estimation of the hydraulic pressure build-up (~5 MPa) around osteocytes (Cowin, Gailani et al. 2009, Gailani, Benalla et al. 2009, Gardinier, Townend et al. 2010). The effects of hydraulic pressure on cells are complicated by spatial heterogeneity in local cell stiffness; for example, the cell body may deform more than cellular processes under hydraulic loads, since the cell’s body is more compliant than its processes (Docheva, Padula et al. 2008). Poroelastic cell models with idealized geometries have been developed to estimate local deformation of a cell under cyclic hydrostatic pressure (Zhang 2005). The primary cilia is a structure that has the potential to sense pressure difference across the cell wall and induce changes in cell behaviours (Bell, Kayser et al. 2008).

2.2. Responses of bone cells to mechanical loading

2.2.1. Intracellular calcium ion concentration

Increase in intracellular calcium ion concentration ([Ca^{2+}]_{i}) is one of the earliest responses of bone cells to mechanical load (Hung, Pollack et al. 1995). It occurs due to both the entry of extracellular calcium and the release of intracellular calcium (Hung, Allen et al. 1996). When rat osteocytes were subjected to membrane stretch with bound \( \alpha V \beta 3 \) integrins, intracellular calcium concentration significantly increased (Miyauchi,
Gotoh et al. 2006). To investigate the ability of bone cells to exchange information through gap junctions under mechanical load, osteoblast-like (MC3T3-E1) cells were seeded on coverslip with micro-patterned coating to force cultured cell to assume a grid-like pattern (Guo, Takai et al. 2006). Then a single cell was mechanically stimulated using atomic force microscopy. Intracellular calcium fluctuations were first observed in a single stimulated bone cell, and then the calcium fluctuation was observed in adjacent cells in the micro-patterned network. Hemichannels and gap junctions play a role in this signaling event (Loiselle, Jiang et al. 2013).

Chicken calvaria primary osteocytes and osteoblast were subject to steady fluid flow (1.2 Pa for 2.5 minutes), and then calcium responses were measured (Kamioka, Sugawara et al. 2006). Osteocytes were less responsive then osteoblast. But in contrast to osteoblasts, calcium responses in osteocytes were immune to Arg-Gly-Asp containing peptides (GRGDS) disruption of focal adhesion, most likely because osteocytes had very low expression of vinculin (Kamioka, Sugawara et al. 2006). These results suggest that osteocytes and osteoblast sense fluid flow-induced shear stress through different mechanoreceptors.

2.2.2. Cytoskeleton

The cytoskeleton in cells provides structure to the cells, imparting rigidity in a fluid-filled construct. The cytoskeleton also provides anchorage for force-generating components of the cells, which in turn controls cell morphology and migration. The
cytoskeleton is also vital in cell signalling and metabolism, since signalling molecules are transported along the cytoskeleton. Two proteins, actin and tubulin, form the bulk of the cytoskeleton in eukaryotic cells. Actins form filament structures, also called stress fibres; and tubulins form tubular structures called microtubules. Together, it is theorized that the actin filaments and microtubules form a load-bearing structure based on the tensegrity theory (Ingber 1997). Conversely, external mechanical loading could alter the cytoskeleton as the cells adapt to this stimulus.

In osteoblast-like (MC3T3-E1) cells, in the physiological range applied for 1 hour, steady flow induced formation of stress fibres (made of mainly actin), while oscillatory fluid flow did not (Malone, Batra et al. 2007). However Ponik et al. (Ponik, Triplett et al. 2007) showed that stress fibres do form in MC3T3-E1 cells, but they need to be subjected to oscillatory fluid flow for at least 5 hours. While in osteocyte-like (MLO-Y4) cells, 24 hours of steady flow was needed to elicit stress fibre formation; after 24 hours of oscillatory fluid flow the number of dendritic processes increased. Under unidirectional fluid shear stress osteocyte-like (MLO-Y4) showed increased dendricity and elongation of dendrites that depends on E11 protein (Zhang, Barragan-Adjemian et al. 2006).

The effect of cyclic hydraulic pressure (CHP) and SHP on osteoblast-like cells (MG-63) has been studied by Tasevski et al. (Tasevski, Sorbetti et al. 2005). The hydrostatic pressure was varied between 0 – 0.8 MPa. The CHP was applied for 1 minute on, then 14 minutes off, for durations from 4 to 12 hours. The mRNA levels for matrix metalloproteinase-1 and -3 (MMP-1 and MMP-3) were significantly increased (p <
0.001) in cells exposed to CHP under serum-free conditions for 4–12 h. mRNA levels for MMP-3, but not MMP-1, were significantly enhanced in cells subjected to SHP. The changes in MMP-1 and MMP-3 may indicate extra cellular matrix (ECM) turnover, and remodelling of bone. The different responses to CHP and SHP suggest that cells have different mechanosensing pathways for CHP and SHP, some of which are responsible for detecting oscillatory patterns in pressure loading. Also the different responses may lead to bone remodelling that result in different bone structure and composition.

MLO-Y4 osteocytes showed increased force traction on beads attached to the cell body, up to 30 pN. The force was enough for the activation of integrins, suggesting a mechanical feedback loop. Experiments with an atomic force microscope have shown increased elastic modulus in osteocytes after mechanical loading (Zhang, Liu et al. 2008). This stiffening response was related to changes in material properties of the cell, suggesting that the cells actively change their cytoskeleton in response to a mechanical load.

You et al. (You, Cowin et al. 2001) suggested that drag forces on the osteocyte pericellular matrix in response to fluid flow could be coupled to, and amplified by, the actin cytoskeleton. Tethering elements, presumably composed of integrins, connect the canalicular wall and pericellular matrix to the osteocyte cytoskeleton (You, Weinbaum et al. 2004). Reilly et al. (Reilly, Haut et al. 2003) showed that degrading the pericellular matrix of osteocyte-like cells diminished the release of prostaglandins following fluid flow exposure, suggesting a role in cytoskeleton-mediated mechanotransduction.
2.2.3. Wnt/β-catenin signalling pathway

The Wnt/β-catenin signalling pathway is vital in osteoblasts for differentiation, proliferation and the synthesis extracellular matrix. Osteocytes have been shown to use the Wnt/β-catenin pathway to transmit signals of mechanical loading to cells on the bone surface (Hartmann 2006). The Wnt/β-catenin pathway in osteocytes may be triggered by crosstalk with the prostaglandin pathway in response to mechanical stimuli, which then leads to a decrease in expression of negative regulators of the pathway such as Sost and Dkk1 (Bonewald and Johnson 2008). Normally the free intracellular levels of β-catenin are kept low by a degradation complex. But mechanical stimuli may cause the collapse of the degradation complex and the release of β-catenin into the cytoplasm where it can subsequently translocate into the nucleus to affect gene transcription. Mechanical loading of the ulna of mouse results in activation of β-catenin signalling 1 h after a loading session, which was only detectable in the osteocytes (Bonewald and Johnson 2008). Fluid flow shear stress induced β-catenin nuclear translocation in MLO-Y4 osteocytes (Kamel, Picconi et al. 2010).

2.2.4. Prostaglandin

Prostaglandins (PGEs, especially PGE2) have multiple effects on bone, including stimulating both resorption and formation (Raisz, Pilbeam et al. 1993, Bergmann and Schoutens 1995). Pulsatile fluid flow (0.64 Pa at 5 Hz for 10 min) enhancement of PGE2 response in MLO-Y4 osteocyte-like cells depends on an intact cytoskeleton, while PGE2
response in MC3T3-E1 osteoblast-like cells was independent of cytoskeleton (McGarry, Klein-Nulend et al. 2005). Oscillatory fluid flow increases PGE2 release in MLO-Y4 cells through ATP mediated pathway, but independent of hemichannel activation (Genetos, Kephart et al. 2007). Oscillatory fluid flow also increased PGE2 expression in osteoblast-like (MC3T3-E1) cells (Malone, Batra et al. 2007). Also cyclooxygenase-2 (COX-2), which involves in the production of PGE2, was upregulated in osteoblast-like (MC3T3-E1) and osteocyte-like (MLO-Y4) cells after extended period (24 hours) of both unidirectional and steady fluid flow (Ponik, Triplett et al. 2007).

2.2.5. RANKL/OPG signalling

Bone resorption, one of the early events in bone remodelling, is mediated by osteoclasts. Two key molecules have been found to mediate osteoclast activity during bone resorption: receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) and osteoprotegrin (OPG). RANKL stimulates osteoclast precursors to commit to the osteoclastic phenotype. OPG binding to RANKL blocks osteoclastogenesis, and decreases the survival of pre-existing osteoclasts. Therefore the rate of bone resorption is dependent on the RANKL/OPG ratio (Boyle, Simonet et al. 2003).

Abundant observations suggest that osteocytes sense mechanical stimuli applied to the skeleton and regulate load-induced remodelling responses, an ideal role for them due to their abundance and location. Another area of significant interest is the
investigation of the mechanisms by which osteocytes communicate with effector cells such as osteoclasts, osteoblasts, and their precursors to regulate bone remodelling.

In a transgenic mouse model in which specifically ablation of osteocytes increased RANKL/OPG ratio in bone, suggesting osteocyte is responsible for the regulation of the RANKL/OPG ratio, and consequently bone resorption (Tatsumi, Ishii et al. 2007). In mice, fluid flow-induced shear stress caused increased RANKL/OPG ratio produced by osteocytes, followed by increased number of osteoclasts and bone resorptive activities (You, Temiyasathit et al. 2008).

2.2.6. Osteocyte viability and apoptosis

CHP has been shown to increase osteocyte viability in calve bone explant study (Takai, Mauck et al. 2004). Bone explants were subjected to CHP with peak of 3 MPa at 0.33 Hz, with a triangle waveform for 1 h/day, starting on day 2. Bone cells were harvested on days 2, 8, 15, and 22 (n = 4). By day 8, CHP increased osteocyte viability compared to controls. In osteoblast-seeded cores, CHP loading also resulted in a trend of increased osteoblast function in the presence of osteocytes (Takai, Mauck et al. 2004). This strengthens the view that osteocytes function as mechanosensors that modulate osteoblast activity. Increased proliferation was observed in rat bone marrow-derived osteoblast-like cells under CHP (0.3 – 5.0 MPa at 1 Hz) (Walboomers, Elder et al. 2006). These cells were from young (12 day old) and old (1 year old) rats, and were seeded onto 3D titanium-mesh scaffold.
In contrast to enhanced viability after stimulation with mechanical loading, osteocyte apoptosis has been linked with increased bone resorption \textit{in vivo} following ovariectomy (Emerton, Hu et al. 2010). It has also been shown that osteocyte apoptosis controls activation of intracortical resorption in the case of bone fatigue (Cardoso, Herman et al. 2009). Therefore osteocyte apoptosis may also be a potent regulator of bone remodelling.

#### 2.3. Micro-cracks and remodelling \textit{in vivo}

Micro-cracks are accumulated in bone during normal physiological loading (Frost 1960). Repetitive loading of the rat ulna has shown the appearance of the basic multicellular unit (BMU) after induction of micro-cracks (Kidd, Stephens et al. 2010). From the same study, the mRNA expressions of remodelling-related genes such as COX-2, OPG, OPN, and RANKL/OPG ratio were changed as soon as 4 hours after micro-crack formation as measured from whole bone extract. Interestingly, the expression levels of some inflammation-related genes such as interleukin-6 (IL-6) and tumour necrosis factor \(\alpha\) (TNF\(\alpha\)) were changed as well.

Micro-cracks in bone are most likely sensed by the osteocytes embedded in the bone matrix. Micro-cracks in the calcified matrix have been shown to be able to rupture osteocytes (Hazenberg, Freeley et al. 2006). These ruptured osteocytes may release signalling molecule to signal bone remodelling directly, or indirectly through surrounding osteocytes by causing them to undergo apoptosis. It is known that the remodelling in
response to micro-crack formation requires apoptotic osteocytes (Cardoso, Herman et al. 2009). However, it appears that without mechanical loading, micro-cracks alone cannot induce bone remodelling (Waldorff, Christenson et al. 2010). Therefore micro-crack induced bone remodelling is likely a process regulated by osteocytes, and this regulation is dependent on mechanical loading.

Figure 2-1. Left panel: A confocal microscopy image of a crack and three osteocytes (solid arrows) with actin staining. The crack (A) has travelled through the lacuna of one osteocyte (B), which appears to have remained intact. The majority of the cell processes are broken by the crack, but some processes seem to cross the crack faces (circle). Right panel: A confocal microscopy image of ruptured cell processes near the origin of the crack (scale bar 50 mm). The crack face displacements in this region were sufficiently large to rupture the cell processes. Adapted from (Hazenberg et al. 2006).

2.4. **In vitro cellular physical damage model**

One *in vitro* cellular damage model has been developed for MLO-Y4 osteocyte-like cell line to simulate the effect micro-cracks (Kurata, Heino et al. 2006). In this model, the cells were seeded in collagen gel. A 21-gauge needle was then used to cause
damage to the gel and the embedded cells. In this study, the damaged MLO-Y4 cells secreted increased levels of macrophage-colony stimulating factor (M-CSF) and RANK; and induced TRACP expression in co-cultured bone marrow cells. A latter study, using the same model, has shown increased RANKL and decreased OPG secretion (Mulcahy, Taylor et al. 2011).

2.5. Mechanical loading systems

2.5.1. Fluid flow shear stress

The majority of studies done on the effect of fluid flow shear stress on bone cells used a parallel plate flow chamber first developed by Frangos et al (Frangos, Eskin et al. 1985). This chamber design was subsequently adopted by researchers to study mechanotransduction of bone cells in vitro (Klein-Nulend, Van der Plas et al. 1995, Jacobs, Yellowley et al. 1998, You, Temiyasathit et al. 2008). Commercial devices with similar designs are also available (Banes, Link Jr et al. 1990, Blackman, Barbee et al. 2000), which offer high through-put capabilities and robust design.

2.5.2. Hydraulic pressure

The effect of cyclic hydraulic pressure on bone cells has been seldom studied in vitro. A number of systems were developed for this purpose. Some have been developed for short-term studies (Parkkinen, Lammi et al. 1995, Pugin, Dunn et al. 1998, Nagatomi,
Arulanandam et al. 2002, Gardinier, Majumdar et al. 2009), while others have developed for long-term application of hydraulic pressure (Sumpio, Widmann et al. 1994, Hasel, Dürr et al. 2002, Watanabe, Inagaki et al. 2005, Maul 2007, Myers, Rattner et al. 2007). Aside from the system used in one study (Liu, Zhao et al. 2010), real-time observation of the cells was not performed. Also, a system that could apply cyclic hydraulic pressure with high throughput capability and robust design does not exist today.
Chapter 3

3. Hypotheses and objectives

3.1. Hypothesis

Osteocytes damaged by physical trauma similar to that induced by micro-cracks would produce increased levels of inflammatory molecules such as PGE2, angiogenic signals like VEGF in addition to remodelling-related signalling molecules COX-2, VEGF, RANKL and OPG. Also, fluid shear stress and hydraulic pressure on osteocytes, either alone or in combination, would alter their response to physical damage. The signalling molecules produced by osteocytes subjected to physical damage and mechanical loading will be able to induce osteoclast formation, and increase the activity of osteoclasts and osteoblasts.

Higher than normal glucose level would adversely affect the ability of osteocyte to respond to mechanical loading in terms of apoptosis, release of inflammatory signal PGE2, and release of RANKL, which controls osteoclastogenesis.

3.2. Objectives
The overall objective of this thesis was to study how combinations of physical trauma, mechanical loading, and chemical factors affect osteocyte response to mechanical loading in terms of apoptosis, release of signalling molecules, and their capacity to affect osteoclast differentiation. The results add to the knowledge of how osteocytes maintain bone homeostasis in the perspective of how they sense micro-cracks in bone and signal to other cells to repair this damage through soluble factors.

3.2.1. Objective 1: Study osteocyte response to subcellular physical damage and mechanical loading in vitro

1. Develop in vitro model for osteocyte physical damage to simulate the physical damage experience by osteocytes

2. Validate the in vitro cellular physical damage model by measuring spatial distribution of cell viability and VEGF expression

3. Identify which osteocyte signalling molecule(s) were affected by physical damage

4. Measure osteocyte synthesis and secretion damage sensitive signalling molecules in the media after they have been subjected to physical damage and mechanical loading in the form of oscillatory fluid shear stress

3.2.2. Objective 2: develop mechanical loading system to simulate the concurrent fluid shear and pressure forces in vitro
1. Design biocompatible hydraulic system that is able to supply 5 MPa of pressure and 5 Pa of fluid shear stress

2. Design biocompatible chamber that is able to withstand 600 kPa of pressure and has geometry to allow the application of laminar flow, while safely contain cells cultured on glass slides

3. Validate mechanical system design through computer simulation

4. Study osteocyte response to concurrent fluid shear stress and hydraulic pressure in terms of apoptosis, actin filament and microtubule organization

3.2.3. Objective 3: study how hyperglycemia affect the capacity of osteocyte to respond to mechanical loading in vitro

1. Develop protocol for subjecting osteocytes to hyperglycemic condition in vitro that is also compatible for applying fluid shear stress in existing flow chambers

2. Measure osteocytes response to mechanical loading (oscillatory fluid shear stress) under hyperglycemic conditions in terms of: a) apoptosis, b) PGE2 release, and c) RANKL release
Chapter 4

4. Effect of mechanical loading on osteocyte response to physical damage

4.1. Introduction

4.1.1. Micro-cracks in bone and osteocytes

Due to the composite nature of bone, microdamage form frequently (Martin 2003). The microdamage are also repaired readily, which maintains the structural integrity of the bone. Recently, it has been shown that remodelling does not occur around microdamage without subsequent mechanical loading (Waldorff, Christenson et al. 2010). However, the cellular mechanism of how subsequent mechanical loading affects the repair of microdamage in bone is not clear.

Microdamage occurs with diffuse damage proceeding primary micro-cracks (Schaffler, Pitchford et al. 1994) (Burr, Forwood et al. 1997) (Frost 1960). Micro-cracks have more impact on bone quality in terms of fracture toughness (Burr, Forwood et al. 1997). Also, only micro-cracks trigger targeted remodelling response by bone cells (Burr, Martin et al. 1985, Bentolila, Boyce et al. 1998, Silva, Uthgenannt et al. 2006), in
contrast to diffuse damages (Herman, Cardoso et al. 2010). In fact, osteonal remodelling preferentially occurs at regions with micro-cracks (Mori and Burr 1993, Bentolila, Boyce et al. 1998). In this thesis, investigation of the cell response to mechanical loading after microdamage would be focused on the effect of micro-cracks.

Repetitive loading of the rat ulna has shown the appearance of the basic multicellular unit (BMU) after induction of micro-cracks (Kidd, Stephens et al. 2010). From the same study, the mRNA expressions of remodelling-related genes were changed after micro-cracks from whole bone extract. The expression levels of some inflammation-related genes such as interleukin-6 (IL-6) and tumour necrosis factor α (TNFα) were changed as well.

The length and width of micro-cracks in bone have been measured in detail. Though the dimension of the crack surface is in the range of hundreds of microns in the longitudinal direction and tens of microns in the transverse direction, the separation of bone matrix and, thus, the cell are in the order of up to 10 microns (Schaffler, Choi et al. 1995, Taylor and Lee 1998, O'Brien, Taylor et al. 2000). Micro-cracks in bone has been thought to initiate repair (Frost 1960), since osteonal remodelling preferentially occurs at regions with micro-cracks (Mori and Burr 1993, Bentolila, Boyce et al. 1998).

4.1.2. Osteocytes physically damaged by micro-cracks in bone

Osteocytes are bone cells distributed throughout the bone (Bonewald 2011). They are essential in normal bone remodelling. Decline in osteocyte density have shown to
result in accumulation of micro-cracks (Vashisht, Verborgt et al. 2000). Osteocyte malfunction (Phillips, Almeida et al. 2008) or absence (Tatsumi, Ishii et al. 2007) in mouse models leads to bone fragility consistent with that observed in aging and osteoporosis. Specifically, while osteocyte ablation increased bone resorption in mice under physiological loading, the same mice were resistant to the disuse-induced bone loss when their hind limbs were subjected to unloading (Tatsumi, Ishii et al. 2007). Due to their location and active role in bone metabolism, osteocytes are suggested to be the ideal cells responsible in sensing and controlling micro-crack-induced remodelling (Parfitt 1994, Verborgt, Gibson et al. 2000, Noble, Alini et al. 2003, Cardoso, Herman et al. 2009). In fact, micro-cracks have been observed to be initiated from lacunae, which house osteocytes (Reilly and Burstein 1975).

Micro-cracks in bone could cause linear damage across osteocyte process or cell body (Hazenberg, Freeley et al. 2006) through the gap created by the discontinuity in the mineralized matrix. In vivo, apoptotic osteocytes were observed near the micro-cracks (Cardoso, Herman et al. 2009).

4.1.3. Existing in vitro physical damage model for osteocytes

Recently, an in vitro study presented a model for osteocyte response to micro-cracks using a needle to physical damage MLO-Y4 cells within a collagen-Matrigel mix; this study has shown that osteocyte promote osteoclastogenesis after 7 days of culture (Kurata, Heino et al. 2006). However, the short term (up to 24 hours) effects of
microdamage on osteocyte are not clear. Also, mechanical loading could not be controllably applied to gel-embedded osteocytes in the previous model, as the fluid flow within the gel matrix could not be controllably applied.

4.1.4. Effect of mechanical loading on osteocytes

Osteocytes are sensitive to mechanical loading with responses including release of soluble factors to initiate bone resorption by osteoclasts or new bone formation by osteoblasts (Noble, Peet et al. 2003, Robling, Bellido et al. 2006, Henriksen, Neutzsky-Wulff et al. 2009, Bonewald 2011). Mechanical loading decreases osteocyte apoptosis induced by TNF-α treatment (Cheung, Simmons et al. 2012). Furthermore, osteocyte apoptosis could induce osteoclast recruitment (Burger, Klein-Nulend et al. 2003, Aguirre, Plotkin et al. 2006, Cheung, Simmons et al. 2012) and differentiation (Al-Dujaili, Lau et al. 2011). Conversely, osteocytes that have been subjected to fluid flow shear stress inhibit osteoclast formation and bone resorption (Tan, de Vries et al. 2007, You, Temiyasathit et al. 2008). One family of mechanically induced molecules are prostaglandins (PGEs, especially PGE2) (Ajubi, Klein-Nulend et al. 1996). They have multiple effects on bone, including stimulating both resorption and formation (Raisz, Pilbeam et al. 1993, Bergmann and Schoutens 1995). Cyclooxygenase-2 (COX-2), which is involved in the production of PGE2 (Ponik, Triplett et al. 2007), is mechanically activated. In osteocyte-like (MLO-Y4) cells, COX-2 was up-regulated after both unidirectional and oscillatory fluid flow (Ponik, Triplett et al. 2007, Kamel, Picconi et al. 2010) and fluid pressure (Liu, Zhao et al. 2010).
Another important molecule in bone remodelling, vascular endothelial growth factor (VEGF), is also expressed in osteocytes. It is upregulated in regions of bone with micro-cracks and correlates with apoptotic osteocytes (Kennedy, Herman et al. 2012). Although it has only been shown to be regulated by mechanical loading indirectly (Cheung, Liu et al. 2011), it may be directly regulated by mechanical loading as well. Interestingly, in vivo studies showed that bone with micro-cracks has increased levels of bone remodelling-related genes COX-2, VEGF (Kidd, Stephens et al. 2010) and RANKL.

Since osteocytes are the major mechanosensor cells in bone (Bonewald and Johnson 2008, Riddle and Donahue 2009), it is plausible that their response to nearby micro-cracks is also mechanically regulated. In fact, it has been shown that micro-crack-induced remodelling requires mechanical loading in vivo, as rats exposed to fatigue loading and subsequently experienced suspension of the loaded limb did not show resorption of damaged bone after 14 days (Waldorff, Christenson et al. 2010).

4.2. Hypothesis

It is hypothesized that osteocyte damaged by physical trauma similar to micro-cracks in bone would produce increased levels of remodelling-related molecules; and this response is mechanically regulated.
4.3. Objectives

To create an *in vitro* model of physically damaged osteocytes (similar to those in bone with micro-cracks), a system was custom made to achieve sub-cellular physical damage to cells similar to the effect of micro-cracks. The physically damaged cells would then be exposed to fluid flow shear stress. The spatial distribution of cell viability and VEGF expression were characterized. The mRNA expression and concentration of signalling molecules involved in the initiation of bone remodelling as listed above were quantified. Furthermore, the capacity of damaged and mechanically stimulated osteocytes to induced osteoclast differentiation was investigated.

4.4. Methods

4.4.1. Osteocyte cell model

MLO-Y4 osteocyte-like cells (gift from Dr. Lynda Bonewald, University of Missouri) were cultured in α-MEM supplemented with 2.5% fetal bovine serum, 2.5% calf serum, and 1% penicillin-streptomycin (Invitrogen) on type I rat tail collagen-coated plates at 37 °C and 5% CO2. The cells were seeded onto collagen-coated glass slides and allowed to grow to 80% confluence before being subjected to physical damage and fluid flow shear stress loading.
4.4.2. Physical cellular damage model

A custom system was designed and made to apply consistent level of physical damage to cells cultured on a 2D substrate (Figure 4-1). This system consists of a horizontal aluminum bar suspended on top of two T-sections. A self-lubricated carriage was mounted onto the horizontal bar and is free to move in one direction horizontally. The moving carriage has a clamp, which holds an array of Tungsten needles with 1 µm tip size (Roboz Inc.). The vertical position of the clamp is adjustable via an M4 screw, with 0.4 mm pitch. Turning the screw will move the clamp up or down. One turn corresponds to 0.4 mm.

The tungsten needles were used to introduce physical damage to MLO-Y4 cells. The path of the damage is typically 1 – 10 µm in width, and 38 or 75 mm in length (the dimension of glass slide). The cell culture was damaged to have ~15% dead cells. This percentage of non-viable cells was observed in fatigue loaded bone in vivo (Verborgt, Gibson et al. 2000).
Figure 4-1. A rail and carriage system to attach 1 µm tipped needles. It allows for smooth horizontal movement and fine vertical position control. Passage of the needles caused rupture of cell membrane. A) System render in CAD software. B) Screw controls vertical position: 1 turn = 0.4 mm. The array of needles is 4 mm across. C) Microscope photo showing tip dimension of micro-needle. D) Typical damage caused by the system. Arrow 1 indicates a cell that was damaged through the cytoplasm. Arrow 2 indicates a cell that was damaged through the nucleus. Arrow 3 indicates a cell with damaged process. E) Paths of damage on glass slide consist of length-wise and width-wise cuts across the slide.

4.4.3. Cell viability staining and quantification

Cell damage was assessed with Trypan Blue (Sigma) dye method adapted from previous study on MLO-Y4 cell viability (Plotkin, Weinstein et al. 1999). Trypan Blue would penetrate apoptotic and necrotic cells, while excluded by cells with intact
membrane. After cellular damage, the samples were washed once with PBS (Gibco). Then, 0.04% Trypan Blue dye in PBS was applied to the cells on glass slides. The slides were imaged with bright field microscope (Zeiss) under 5x objective. The x, y coordinates of positively stained cells were recorded in each field of view. The damage sites were represented by a linear function, \( y = mx + b \), where \( m \) is slope and \( b \) is y intercept. The distance of each positively stained cell to the closest damage site was calculated. Each field of view had at least 3 damage sites and 1000 cells. The experiment was repeated four times, with at least 5 samples per experiment.

Live/Dead assay (Invitrogen) was performed on damaged MLO-Y4 cells according to manufacture instructions. This assay contains two dyes, Calcein AM and Ethidium homodimer-1. Calcein AM would be converted to fluorescent calcein in live cells. Ethidium homodimer-1 enters cells with damage membrane and would be amplified by binding to nucleic acid. To make the staining solution, Calcein AM and Ethidium homodimer-1 were diluted to 2 \( \mu \)M and 4 \( \mu \)M respectively, in PBS. The staining solution was applied to MLO-Y4 cells on glass slide for 40 minutes at room temperature. Fluorescent microscope (Nikon) was used to image the slides for calcein AM (ex/em \( \sim \)495 nm/\( \sim \)515 nm), and Ethidium homodimer-1 (ex/em \( \sim \)495 nm/\( \sim \)635 nm). The number of cells was counted in each field of view at 20x magnification for each filter. Each field of view had at least 3 damage sites and 1000 cells. The experiment was repeated four times, with at least 5 samples per experiment.

4.4.4. Fluid shear stress treatment
Parallel flow chambers were used to apply oscillatory fluid shear stress to the cells on glass slides as previously described (Jacobs, Yellowley et al. 1998, You, Temiyasathit et al. 2008). The flow chamber creates a flow space above the cells that is 70 mm x 35 mm x 0.4 mm. It is modelled as a parallel plate flow volume. Assuming vicious flow, the shear stress on the cells (wall shear stress) is expressed as $\tau=6\mu Q/(bh^2)$ where $\tau$ is the shear stress; $\mu$ is the viscosity of the medium, which is approximated to be that of the water at room temperature ($1\times10^{-3}$ Pa s); $Q$ is the maximum flow rate during the oscillatory flow, which is 0.5 ml/s as a result of the syringe displacement; $b$ is the flow channel width, which is 35 mm; $h$ is the flow channel height, which is 0.4 mm.

Cell in all conditions were placed into flow chamber for the duration of the experiment. Fluid flow was applied by a 3 ml plastic syringe (BD), which is driven by a linear actuator. The flow was oscillatory with sinusoidal profile at frequency of 1 Hz, and applied peak shear stress of 2 Pa to the cells. This shear stress was applied for 1 hr. Then the slides were taken out from the flow chamber and incubated with fresh media for 23 hrs. The time line is shown in Figure 4-2. This experiment was repeated for at least three times for each assay.

![Figure 4-2. Time line of combinations of fluid shear and physical damage experiment.](image)
4.4.5. mRNA expression quantification

Total RNA was isolated from MLO-Y4 cells at 6, 8 and 24 hours after flow treatment. Reverse transcription was performed with Superscript III (Invitrogen). The resulting cDNA samples were subjected to quantitative PCR with SYBR Green I (Roche) for genes IL-6, TNFα, COX-2, VEGF, RANKL, and OPG. Mastercycler ep realplex2 (Eppendorf AG) was used to run the Real-time PCR machine. Expression levels of each gene were normalized to the housekeeping gene 18s.

4.4.6. Protein expression quantification

Levels of VEGF and PGE2 were measured in the media from the post-flow incubation (24 hours). VEGF was measured with ELISA kits (Mouse VEGF ELISA Duo Set, R&D Systems). PGE2 levels were measured with EIA kits (Cayman Chemical).

4.4.7. VEGF immunostaining

After being subjected to physical damage using the system developed in this study, MLO-Y4 cells on glass slides were incubated for 30 minutes. They the cells were fixed with 3% paraformaldehyde for 15 min. PBS was used as wash buffer for three times at 5 minutes each for each wash. The cells were blocked and permeabilized with 1% BSA in 0.1% PBS-Tween 20 for 1 hour. Rabbit anti-mouse VEGF antibody was diluted in the blocking/permeabilization buffer to 5 μg/ml, and applied to the cells for 16 hours at 4°C.
Goat Anti-Rabbit fluorescently labelled antibody was diluted in the blocking/permeabilization buffer to 10 μg/ml, and applied to the cells for 1 hour at room temperature. The nuclei were lastly labelled with DAPI.

The cells were imaged with a fluorescent microscope. Three separate fields of view were taken per slide. Images from each fluorescent channel were saved as gray scale tif files. The intensity of VEGF staining in each cell was quantified by measuring the integrated density of the cell in ImageJ. The background intensity was calculated by multiplying the mean intensity of three adjacent empty regions by the cell area. This background intensity was subtracted from the integrated intensity of each cell to correct for background.

4.4.8. Osteoclast differentiation of RAW 264.7 in conditioned media and TRAP staining

RAW264.7 cells were cultured in growth media until 70% confluence. The growth media consists of DMEM (Sigma) with 10% FBS (Gibco), 1% P/S (Gibco), and 4 mM L-glutamine (Sigma). Then the cells were cultured in differentiation media for 7 days. The differentiation media was growth media with 25 ng/ml RANKL (R&D Systems), which was mixed 1:1 with conditioned media from MLO-Y4 cells that had been subjected to combinations of physical damage and fluid shear. Media was changed daily. After 7 days of differentiation, the cells were fixed and stained for tartrate-resistant
acid phosphatase (TRAP), which is only expressed in osteoclasts. TRAP positive cells with three or more nuclei were quantified under bright field microscope (Zeiss).

4.4.9. Statistical Analysis

All experiments were repeated for at least 3 times, with 4-6 replicate samples per condition. Factorial ANOVA is used to investigate the interaction effect of fluid flow and physical damage. In experiments with two stimuli: fluid flow shear and physical damage, a $2^2$ factorial design was used to investigate the effect of individual stimulus as well as their interactive effects. The statistical software JMP was used for this analysis.

4.5. Results

4.5.1. Physical cellular damage model validation

The capability of the physical cell damage system was assessed through observation of damaged cells, and viability staining. The cell damage system was able to damage MLO-Y4 cells cultured on glass slides. The linear damage was induced across the glass slides (75 mm x 38 mm) as shown in Figure 4-1E. The damage site width ranges from 1-10 µm. Since the in vivo cultured cells are randomly distributed over the slides, different parts of the cells were damaged depending on their relative position to the damage site. Various parts of the cell were cut through during the damage process, including nucleus, part of the cell body, and the processes (Figure 4-1D). A significant
number of dead cells were observed near the damage sites using Trypan Blue and Live/Dead assay (Figure 4-3A, B). Most Trypan blue stained cells were within 150 µm of the nearest damage site (Figure 4-3C). The density of Trypan blue stained cells was significantly higher within 150 µm of the damage site compared to area that is further away (Figure 4-3D).

Figure 4-3. A) Trypan Blue staining showed uptake of the dye near the damage sites (labeled with lines). The inset shows a magnified area with stained (blue) and non-stained cells B) LIVE/DEAD staining of damaged MLO-Y4 cells was used to quantify the percentage of cells that have sustained damage. Live and dead cells are labeled green and red respectively. The inset shows a magnified area with live and dead cells. C) A representative plot of six samples for the number of cells as a function of distance to the damage site. D) Trypan Blue-positive cell density was quantified vs. distance to closest damage sites. Data shown are mean ± standard deviation. (* P < 0.05, A scale bar = 50 µm, B scale bar = 250 µm)
4.5.2. mRNA expression of genes associated with remodelling changed 24 hours after physical cell damage

Changes in transcription of remodelling-related genes: IL-6, TNFα, COX-2, RANKL, and OPG were measured by quantifying their mRNA levels. From all cells in each sample, COX-2 and VEGF mRNA levels significantly increased only at 24 hours after cell damage (Figure 4-4). RANKL, OPG, IL-6, TNF-α mRNA levels were not changed at the time points tested (See Appendix A-1). From each individual stimulus, the increase in COX-2 and VEGF was over 1.5 fold over control. Concurrent fluid flow shear stress and physical damage induced greater than 3 fold increase in COX-2 and VEGF mRNA over control (Figure 4-5) 24 hours after physical damage to the cells.

![Graphs showing mRNA levels of COX-2 and VEGF over time](image)

*Figure 4-4. Time-point response of MLO-Y4 cells to physical damage without fluid shear stress (n = 6). mRNA levels of VEGF and COX-2 are elevated 24 hours after damage in MLO-Y4 cells. Data shown are mean ± standard deviation.
4.5.3. Concentration of PGE2 and VEGF in media 24 hours after physical cell damage

The concentration of PGE2 and VEGF in media showed similar level of increase to the corresponding gene expressions for COX-2 and VEGF at 24 hours after cell damage. With only physical damage, the increases in PGE2 and VEGF were not significantly different from control. With only fluid flow shear stress, PGE2 was not significantly different from control; VEGF showed a 2-fold increase. Concurrent fluid flow shear stress and physical damage induced 5-fold increase in PGE2, and 3-fold increase in VEGF over control (Figure 4-6).
4.5.4. VEGF expression in damaged and adjacent MLO-Y4 cells

The location and change of VEGF production in osteocytes are indicative of angiogenesis. While VEGF is ubiquitously expressed in MLO-Y4 cells, its expression was locally elevated near the damage sites (Figure 4-7A). The intensity of VEGF staining could be as high as 2.6 times higher compared with the baseline expression. The elevated VEGF expression drops as a function of the distance to the damage sites (Figure 4-7B). For cells more than 150 μm from a damage site, the VEGF expression had dropped to baseline level. Cells that were within 150 μm from a damage site had over two-fold increase in intensity of VEGF (Figure 4-7C).
4.5.5. Effect of damaged osteocyte conditioned media on osteoclasts differentiation

Osteoclasts are the effector cells that resorb bone. The differentiation of osteoclasts from precursor cells by the conditioned media is a functional indicator of remodeling signals. RAW264.7 osteoclast precursor cells were cultured with conditioned media from MLO-Y4 cells that were subjected to either fluid flow shear stress and/or
physical damage. TRAP positive cells are most numerous in the control group, lower in the physically damaged group, and very low in the flow only and flow + damage groups (Figure 4-8). Media from damaged MLO-Y4 cells produced a threefold reduction in osteoclast number. Fluid flow shear stress conditioned media abolished the formation of osteoclasts. The combination of physical damage and fluid flow shear stress induced increased number of osteoclast comparable to fluid flow shear stress only, but the difference in means was not statistically significant.
Figure 4-8. A) TRAP staining of differentiated RAW 264.7 cells cultured in conditioned media from MLO-Y4 cells with combination of physical damage and fluid flow shear stress. Horizontal and vertical labels indicate the stimuli applied to MLO-Y4 cells, from which the conditioned media was used to culture RAW 264.7 cells. Scale bar = 100 μm. Inset showing multinucleated TRAP+ cells. B) Quantification of TRAP staining of RAW 264.7 cells differentiated with MLO-Y4 conditioned media. The number of cells that are TRAP+ and has 3+ nuclei was counted. Means that do not share a letter are significantly different. Data shown are mean ± standard deviation.
4.6. Discussions

In this study, a robust *in vitro* cellular physical damage model was developed to allow the application of controlled fluid shear mechanical loading to physically damaged osteocytes for measurement of short term (24 hours) response. Specifically, to simulate the physical damage of micro-cracks on osteocytes comparable to what could occur *in vivo* (Hazenberg, Freeley et al. 2006), we have designed and assembled a system that allowed us to induce arrays of linear damage to the cells at the sub-cellular scale. The damage sites produced using this device are in the range of 1 to 10 microns. The damage induced by our device was able to cause sub-cellular damage to the MLO-Y4 osteocytes cultured on glass slides. A linear damage profile was used in the study as it approximates the shape of *in vivo* micro-cracks. The damage profile with respect to the cell components is indiscriminate. The effectiveness and validity of our *in vitro* physical damage model were measured by the creation of spatially distinct pattern of the osteocyte cell death and expression of VEGF. This model reproduced the loss in cell viability as seen from *in vivo* observations (Verborgt, Gibson et al. 2000, Cardoso, Herman et al. 2009, Kennedy, Herman et al. 2012). With this system, we showed that osteocyte response to physical damage is mechanically regulated, indicating mechanical loading plays a critical role in the repair of micro-cracks through targeted bone remodelling. We have observed that mechanical loading in the form of fluid flow shear stress dramatically increased the level of remodelling initiation signals produced by osteocytes.
4.6.1. Comparison with existing *in vitro* cellular physical damage model

Previous study had shown an *in vitro* cell damage model for MLO-Y4 osteocyte-like cell line (Kurata, Heino et al. 2006). In this model, the cells were embedded in collagen-Matrigel mixture. A 21-gauge needle was then used to cause damage to the gel and the embedded cells. With the cells embedded in gel, controlled fluid flow shear loading could not be applied to the cells. In this study, a new damage system was developed in this study that allows application of fluid flow shear stress to the cells either before or after physical damage.

Kurata et al., have reported that the damaged MLO-Y4 cells secreted increased levels of macrophage-colony stimulating factor (M-CSF) and RANKL and induced TRACP expression in co-cultured bone marrow cells. A latter study, using the same model, has shown increased RANKL and decreased OPG secretion (Mulcahy, Taylor et al. 2011). We have not seen similar increase in RANKL. This is most likely due to time point of the observation. We have shown a model for short term (24 hours) response by osteocyte here. Whereas with the previous model (Kurata, Heino et al. 2006), the osteocyte media was analyzed after 7 days of culture and intermittent physical damage each day.

4.6.2. Comparison with fracture-induced effects
It could be argued that the damaged osteocyte population produced by our system could be a model for osteocytes in bone fracture. However we have controlled the viable osteocyte population to be 86% - 84%, while osteocyte viability in long bone (femur) fracture is from 25% to 58% (Dunstan, Somers et al. 1993). In fatigue loaded rat long bone, osteocyte viability is around 80% (Verborgt et al. 2000), very close to our condition. This is in contrast to healthy bone tissue, which has more than 95% viable osteocytes (Dunstan et al. 1993).

4.6.3. Comparison of loss of cell viability versus in vivo data

Fatigue loading of long bone in a rat model has induced a loss of osteocyte integrity localized to the region of bone containing micro-cracks (Verborgt, Gibson et al. 2000). It has been shown that the osteocytes around the micro-crack would undergo apoptosis (Verborgt, Gibson et al. 2000). A more recent study validated this finding in a more quantitative manner: as a function of distance to the damage site (Kennedy, Herman et al. 2012). The cell damage system presented in this thesis had the capability to create a spatial distribution of dead cells (Figure 4-3 C, D) closely resembling that of the in vivo fatigue loading study, which has shown elevated cell death in the range of 150 µm from the damage site. In a rat long bone model, early loss of osteocyte integrity and osteocyte apoptosis as a result of micro-cracks have been shown to be initiators of remodelling in fatigue loaded bone (Cardoso, Herman et al. 2009). Osteocyte apoptosis is well known to be critical in the initiation of bone remodelling (Aguirre, Plotkin et al. 2006, Al-Dujaili, Lau et al. 2011, Cheung, Liu et al. 2011, Cheung, Simmons et al. 2012). The inhibition
of osteocyte apoptosis is also well studied (Aguirre, Plotkin et al. 2005, Plotkin, Mathov et al. 2005). Furthermore, osteocytes that are affected by micro-cracks may experience necrosis as well as apoptosis. But in either case, osteocytes with compromised integrity have been associated with bone remodelling around micro-cracks (Verborgt, Gibson et al. 2000).

4.6.4. Distribution of VEGF protein expression in MLO-Y4 osteocytes subjected to physical damage

The importance of VEGF to bone remodelling had been highlighted in the case of fracture repair (Ito et al., 2005). VEGF is a crucial rate-limiting signal in angiogenesis; also it increases vascular permeability, possibly allowing progenitors to enter into the remodelling site (Ferrara, Gerber et al. 2003). In this way, VEGF is required in the initiation of the BMU (Smith and Calvi 2013). Also VEGF has been shown to regulate osteoblast survival (Street and Lenehan 2009). Therefore, the spatial distribution of VEGF expression level is critical to the directionality of the targeted bone remodelling process. Through staining of intracellular VEGF, we have found that the expression of VEGF protein in cell cytoplasm increased around the damage sites produced by the device used in this study approximate that produced in vivo (Kennedy, Herman et al. 2012) in terms of the distance of high expression level relative to the damage site. Up-regulation of VEGF mRNA and consequently, medium concentration levels would have been significant in creating a gradient to direct angiogenic sprouting (Shin, Jeon et al. 2011).
4.6.5. Mechanical loading and physical damage had interactive effect on expression of PGE2, VEGF and associated gene expressions

In fracture repair, COX-2 and consequently PGE2 have been shown to be vital for remodelling (Xie et al., 2008). Concurrent stimuli of fluid shear stress and damage increased osteocyte expression of inflammatory (COX-2) and angiogenic (VEGF) genes, as well as the corresponding released molecules (PGE2, VEGF), which are higher than that from each stimulus alone. The PGE2 level (~ 8000 pg/ml) from the combination of fluid flow shear stress and physical damage was in the range (> 3520 pg/ml) to cause osteogenic effect in bone marrow stromal cells, whereas the PGE2 levels in other groups were not (Keila, Kelner et al. 2001). The PGE2 concentration has increased nearly 2 fold under fluid shear stress versus control. Though not statistically significant, the trend in our PGE2 data agree with previous studies on the effect of fluid shear on MLO-Y4 cells (Bonewald 1999, Kamel, Picconi et al. 2010) as well as primary osteocytes (Ajubi, Klein-Nulend et al. 1996). PGE2 concentration is sensitive to duration as well as magnitude of loading, and the relationship has shown to be non-linear (Kamel, Picconi et al. 2010). The discrepancy between the PGE2 concentration we have observed and other studies was likely caused by the time point at which the media PGE2 level was measured.

VEGF could be induced by PGE2 in osteoblasts (Harada, Nagy et al. 1994). Also, PGE2 has been shown to be upstream of the β-catenin pathway, which controls VEGF
expression in osteocytes (Kitase, Johnson et al. 2007). Conversely VEGF promotes late COX-2 protein induction (Clarkin, Emery et al. 2008), which may explain the result of increased in COX-2 mRNA and PGE2 concentration 24 hours after treatment observed in this study. The coincidental increase in both PGE2 and VEGF at the 24 hour point hints at a positive feedback loop that increases the concentration of both signals in preparation for establishing the BMU.

4.6.6. Time-dependency of remodelling related signals

Soluble RANKL and OPG mRNA levels did not change in response to physical damage in the time point (24 hours) used in this study. But RANKL has been shown to increase in MLO-Y4 cells after 7 days of physical damage (Kurata, Heino et al. 2006). From these results, osteocyte signalling in response to physical damage is time sensitive. On the other hand, IL-6, TNF-α mRNA levels did not change in response to physical damage, suggesting that osteocytes may not be responsible for the changes in these genes that were observed within 24 hours of bone fatigue damage in vivo (Kidd, Stephens et al. 2010).

4.6.7. Possible effect of physically damaged osteocytes on osteoclast differentiation
Cracks in bone tend to propagate from existing cracks (Bonfield, Grynpas et al. 1978), where existing remodelling may be already taking place (Waldorff, Christenson et al. 2010). Also, micro-cracks tends to form ahead of propagating cracks (Vashishth, Tanner et al. 2000). Osteocytes signalling in response to micro-cracks may have an effect on existing remodelling processes. Specifically, osteoclast differentiation may be affected by damaged osteocytes. In this study, RAW264.7 cells differentiated into TRAP+ cells in conditioned media from MLO-Y4 cells that are subjected to combinations of physical damage and fluid shear. Signals from osteocytes subjected to fluid shear stress reduced the number of osteoclasts, as has been well established in the literature (Tan, de Vries et al. 2007, You, Temiyasathit et al. 2008). Physical damage also reduced the number of osteoclasts compared to the control, but not to the same degree as fluid shear (Figure 4-8B). Osteocyte response to mechanical loading seems to have a dominant effect on osteoclast differentiation, since media from osteocytes subjected to combinations of physical damage and shear stress has reduced the number of osteoclast to the same level shear stress alone (Figure 4-8B). Damage by itself on the MLO-Y4 cells induced an intermediate number of osteoclast between control and shear stress groups (Figure 4-8B). It is possible that physical damage and/or fluid shear could affect signals such as M-CSF that affect proliferation of RAW264.7 cells. At the time point of 3 days after damage, M-CSF was not altered by micro-cracks in vivo (Kennedy, Herman et al. 2012). However this does not rule out other signals that support monocyte proliferation, such as plasminogen activator inhibitor, which has not been investigated in osteocytes. Another possibility is the time point chosen for the osteocyte media collection, which is 24 hours after damage. Our results points to an inhibitory effect of physically damaged osteocytes
on osteoclast differentiation shortly (24 hours) after the onset of micro-crack. In contrast, the evidence of RANKL production by osteocyte near micro-cracks is obtained for 3-day (Kennedy, Herman et al. 2012) and 7-day (Kurata, Heino et al. 2006) time points. Taken together, the inhibitory effect of micro-cracks on osteoclastogenesis may be transient, and only occurs shortly after micro-crack formation.

4.6.8. Osteocytes are the likely cell type in bone that are subjected to physical damage

It is not known whether the observed effect of sub-cellular damage in this study is unique to osteocytes. However, osteocytes are the most abundant and, likely, the only cell type in bone that experiences rapid (order of 100 μm/s) matrix dislocation in vivo caused by the formation of micro-cracks (Vashishth, Verborgt et al. 2000), which in turn causes physical damage to osteocytes at the subcellular level. Therefore we have only conducted our damage experiments on MLO-Y4 cells to test our hypothesis.

4.6.9. Limitations

A limitation in our study is the in vitro nature of the experiment. The MLO-Y4 osteocyte cell line express many markers of osteocytes (Bonewald 1999). It also has similar response to mechanical loading (Bonewald and Johnson 2008). But MLO-Y4 cells have different expression levels of many proteins compared with primary cells
(Yang, Harris et al. 2009). The 2D nature of the culturing condition was also very different from the \textit{in vivo} environment. This difference in substrate geometry could affect cell attachment, which consequently affects cell strain at a given flow rate (You, Cowin et al. 2001). In the \textit{in vitro} system, dissolved signalling molecules could also diffused over the culture more readily. This process would have been much slower \textit{in vivo} due to the much lower permeability of the lacuna-canalicular space (Goulet, Coombe et al. 2009). Osteocytes also produce other factor at different stages of bone remodelling, including RANKL, OPG, sclerostin, osteocalcin. Mechanosensitivity of these factors in damage osteocytes should be investigated in the future.

Osteocyte signalling in response to micro-cracks and mechanical loading is a complex and dynamic event in space and time. In this study, we have developed a robust \textit{in vitro} cellular physical damage model, which enables a better understanding of mechanical regulation of osteocyte short term response to micro-crack-induced sub-cellular scale physical damage. The spatial distribution of non-viable cells approximated \textit{in vivo} observations. We showed that the cellular mechanism involved in targeted bone remodelling to micro-cracks includes altered inflammatory and angiogenic gene expression levels in osteocytes; and this response is enhanced by mechanical loading.

\textbf{4.6.10. Future directions}

The signals produced by osteocytes in response to micro-cracks are highly time-dependent. In order to understand how osteocyte controls the bone remodelling in this
case, a wider array of signals would have to be measured in time points of within 6 hours of physical damage. Some of the signals such as PGE2 (Kamel, Picconi et al. 2010), and nitric oxide (Vatsa, Smit et al. 2007) have very high rate of change in response to external stimuli. Equally important are some of longer lasting signals such as RANKL and M-CSF, which are critical in osteoclast formation. In these cases, time point in the order of days would have to be used to study the dynamics of these signals.

Though RANK, TNF-α, and IL-6 was not changed at the time point of 24 hours used in this study, osteoclast differentiation was nonetheless affected by conditioned media from MLO-Y4 osteocytes that have been subjected to mechanical loading and physical damage. This was an unexpected result, since no other known signals produced by osteocyte could significantly affect osteoclast differentiation. Investigation into the conditioned media could lead to discovery new osteocyte signalling factors that control bone remodelling.
Chapter 5

5. Mechanical system capable of concurrent application of oscillatory fluid shear and hydraulic pressure

5.1. Introductions

Hydraulic pressure and fluid flow shear stress are two concurrent mechanical stimuli on osteocytes. They have been shown to be the significant forces in bone remodelling at the tissue level (Klein-Nulend 1990, Qin, Kaplan et al. 2003). The effects of fluid shear alone (Tan, Bakker et al. 2008, You, Temiyasathit et al. 2008) and pressure alone (Rubin 1997, Liu, Zhao et al. 2010) on osteocytes have been studied separately. However, the effects of pressure and flow have not been systematically compared and the effect of both at the cellular level is still unclear.

In bone, movement of the interstitial fluid around osteocytes creates shear stresses in the range of 0 to 20 dynes/cm² (Mi, Fritton et al. 2005).
Under physiological activities, such as walking, hydraulic compression is cyclic in nature. Oscillatory loading of bone with 0-18 MPa at 1 Hz was calculated to induce 0.27 MPa fluid pressure at the lacunar-canaliculAr porosity (Zhang, Weinbaum et al. 1998). The most recent studies found that the hydraulic permeability of the bone tissue was smaller than the previous model assumed, leading to an even higher estimation of the hydraulic pressure build-up (~5 MPa) around osteocytes (Cowin, Gailani et al. 2009, Gailani, Benalla et al. 2009, Gardinier, Townend et al. 2010).

The majority of studies done on the effect of fluid flow shear stress on bone cells used a parallel plate flow chamber first developed by Frangos et al (Frangos et al. 1985). For hydraulic pressure application, a number of systems have been developed for short (Parkkinen, Lammi et al. 1995, Pugin, Dunn et al. 1998, Nagatomi, Arulanandam et al. 2002, Gardinier, Majumdar et al. 2009), and long term (Sumpio, Widmann et al. 1994, Hasel, DÄ¼rr et al. 2002, Watanabe, Inagaki et al. 2005, Maul 2007, Myers, Shrive et al. 2007) studies.

However, a system capable of applying both hydraulic pressure and fluid shear stress is not currently available. Therefore the development of a system with this capability will also be a part of the proposed study.

5.2. Hypothesis

Oscillatory fluid shear stress and hydraulic pressure have an synergistic effect on osteocyte apoptosis and cytoskeleton organization.
5.3. Methods

5.3.1. Cell culture

MLO-Y4 osteocyte-like cells were cultured in α-Modified Eagle’s Medium (Gibco) supplemented with 2.5% fetal bovine serum (Gibco), 2.5% calf serum (Gibco), and 1% penicillin and streptomycin (Gibco) on 100 mm petri dishes coated with type I rat tail collagen. Prior to mechanical loading, the cells were seeded onto collagen-coated glass slides (75 x 38 mm) and allowed to grow to 80% confluence.

5.3.2. Mechanical loading chambers

The geometry of the pressure/flow loading chamber that houses the cells was defined using the computer aided design software SolidWorks. It was used to generate 3D model (Figure 5-1 A, C), engineer drawings of the pressure-flow chamber and finite element analysis of the design. The geometry inside of the chamber is defined to allow approximation as a parallel plate flow space. It has height of 0.6 mm, width of 38 mm, and length of 75 mm. The relationship between flow rate and shear stress could be derived from Navier-Stokes and continuity equations to be \( \tau = \frac{6Q \mu}{bh^2} \), where \( \tau \) is the shear stress; \( \mu \) is the viscosity of the medium, which is approximated to be that of the water at room temperature \( (1 \times 10^{-3} \text{ Pa s}) \); \( Q \) is the flow rate which is 0.5 ml/s as a result of the syringe displacement; \( b \) is the flow channel width; \( h \) is the flow channel height.
Two prototypes were machined and assembled (Figure 5-1 B, D). A design that allows the containment of one slide was made using transparent polycarbonate material. This prototype was used to test cell viability in the geometry of the flow space. It was also used to develop and optimize the assembly and disassembly protocols. The advantage of the transparent material was the direct visualization of fluid within the chamber space. This chamber had slide-in mechanism for loading slides. The side pieces were identical. Each had ports for accommodating connectors. The flow channels were sealed with O-rings made from Viton, which is a bio-compatible material.

A second design that allows the containment of 8 slides was made using additive manufacturing process. The material of this prototype was VeroWhite (PolyJet). It is a biocompatible polymer that has elastic modulus comparable to polystyrene (~ 3 GPa). The fluid flow space was identical to the polycarbonate prototype. Viton O-rings were used to seal the fluid space.
5.3.3. Force application system

The pressure force is applied using a linear actuator, which is crank shaft driven by a geared electric motor. The force is transduced to the fluid using a 1ml plastic syringe. This system is able to deliver up to 5 MPa of hydraulic pressure.

The fluid flow shear stress is applied by a similar linear actuator. The fluid flows in a closed loop with a 1 ml plastic syringe (BD) on either end. Both syringes are driven
by the linear actuator. The syringes are driven such that they are 180 degrees out of phase, i.e. when one pulls, the other pushes. Since the motion of the syringes is driven by a crank shaft, the velocity profile of the fluid is sinusoidal.

A pressure regulator was used to release higher-than-desired pressure. To replenish the media in the circuit in the in event that some are lost during over-pressure, a reservoir separated by a check valve added the circuit as well. Simulation performed in MatLab was used to confirm the pressure profile in this setup (Figure 5-2).

During the experiments peak pressure of 550 kPa was used to maintain the deformation of the chamber to be within acceptable levels. The peak magnitude of the fluid shear was 1 Pa. The oscillatory frequency was 1 Hz. The mechanical loadings were applied for 1 hour during each experiment.
Figure 5-2. The mechanical loading circuit was designed as a circuit diagram A) first. Then the components were abstracted in MATLAB Simulink B). The pressure input and pressure induced in the chamber were computed and graphed C). The physical circuit is shown in D) with a pressure gauge attached for pressure validation. Two linear actuators provided sinusoidal loadings to the cells through back-to-back syringe for fluid shear stress E), and single syringe for hydraulic pressure F). All syringes used had 1 mL capacity.

5.3.4. Cell apoptosis

To assess the effects of combinations of shear stress and hydraulic pressure on osteocytes, apoptosis in MLO-Y4 cells was induced by serum starvation of the cells with α-MEM containing 0.8% FBS, 0.8% CS and 1% P/S for 24 h prior to the application of mechanical loading. After being subjected to mechanical loading for 1 hour, the glass slides with cells were extracted from the chambers and placed in 10 cm circular petri dishes. Fresh 1.6% serum media (0.8% FBS + 0.8% CS) was added to the dish. The cells were incubated for 30 minutes.

The apoptosis level of cells was measured using Apopercentage Assay (Accurate Chemical) as indicated by the manufacture. The apoptotic cells were stained pink.

Apopercentage dye was diluted 1:40 in 1.6% serum media. The cells were treated with the dye and incubated for an additional 30 minutes. Then the samples were washed with PBS to remove the dye.

The percentage of apoptotic cells was measured for each of the four experimental loading conditions. The stained cells on glass slides were imaged under a light microscope (Zeiss). Four different regions were imaged for each slide. The number of apoptotic cells and non-apoptotic cells were quantified using the Cell Counter plug-in of Image J software.
5.3.5. Cytoskeleton reorganization

MLO-Y4 cells were exposed to CHP loading or non-loading (static control) for 1 hour, and then their actin filament and microtubules staining were performed as previously described (Liu et al. 2010) (n = 8). To stain the actin filaments, the cells were fixed with 3.7% paraformaldehyde (Sigma) in PBS for 10 minutes, then permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 5 minutes. The cells were stained with Alexa Fluor 488 phalloidin (Invitrogen). To stain the microtubules, the cells were fixed with 0.25% glutaraldehyde and permeabilized with 0.1% Triton X-100 in PHEM buffer (25 mM HEPES, 60 mM PIPES, 10 mM EGTA, 2 mM MgCl, pH 6.9, warmed to 37°C) for 30 minutes. Following fixation, cells were quenched in 2 µg/ml of sodium borohydride for 15 minutes. The fixed cells were treated with 10% BSA for 1 hour to reduce non-specific binding. Microtubules were first labelled with 2 µg/ml alpha-tubulin antibody (AB Cam) for 3 hours, and then with 50 µg/ml FITC secondary antibody (Invitrogen) for 1 hour. Cells were imaged using a fluorescent microscope.

The local bending sections with high curvature in microtubule have been shown to be the result of localized buckling (Brangwynne, MacKintosh et al. 2006). In this study, the local bending sections with high curvature in microtubules were referred to as buckling regions. The buckling regions in microtubules were quantified using a chord-to-point distance accumulation (CPDA) method (Awrangjeb and Lu 2008), implemented in MatLab. The CPDA program recognizes microtubules and calculates the number of buckling regions and the curvatures of these buckling regions. A square ROI of 150×150
pixels were selected on the microtubule immunofluorescent images of the cell. The ROI’s were selected to exclude the cell nucleus. The number of buckling regions and the curvature normalized with the number of buckling regions were quantified. The buckling regions in the ROI were counted and the curvature at each buckling region were calculated.

5.3.6. Statistics

All experiments were repeated for at least 3 times, with 4-6 replicate samples per condition. ANOVA and Tukey post-hoc were used to determine the significance of differences between the means.

5.4. Results

5.4.1. Validation of cell loading chambers

The deformation of the chambers under hydraulic pressure was calculated using finite element analysis in SolidWorks. The pressure load was modeled as static to investigate the maximum deformation of the chamber material. The polycarbonate single slide chamber had a maximum of 60 μm of deformation at the center of the chamber (Figure 5-3A). The VeroWhite 8-slide chamber had 46 μm of deformation along the long axis of the flow space (Figure 5-3B).
The biocompatibility of the polycarbonate single slide chamber and the 8-slide cell chamber was validated using MLO-Y4 cells. The cultured cells were not disturbed by the assembly and disassembly processes. Also, pressure of 600 kPa for 2 hours did not cause significant deformation in the pressure-flow chamber.

![Material displacement in the direction that is normal to the cell-seeded surface in the polycarbonate single slide chamber A) and 8-slide VeroWhite chamber B).](image)

5.4.2. Apoptosis was reduced by concurrent fluid shear and pressure forces

MLO-Y4 osteocyte cells were subjected to oscillatory fluid shear stress, hydraulic pressure and concurrent loading of both. Static group was used as control, which was placed into the same loading chamber as the loaded groups. The control group had the highest level of apoptosis. Either fluid shear stress or hydraulic pressure alone had
reduced this level by 3-fold. When concurrent fluid shear stress and hydraulic pressure were applied concurrently, the apoptosis level of the cells was reduced by 6-fold.

![Graph showing apoptosis levels under different conditions](image)

**Figure 5-4.** Percentage of apoptotic MLO-Y4 cells that had been subjected to static control condition (C), fluid shear stress (F), hydraulic pressure (P), and concurrent fluid shear stress and hydraulic pressure (P+F).

### 5.5. Discussions

#### 5.5.1. Transport of dissolved gases

The pressure-flow circuit would be fully fluid filled, and would not have significant amount of gas inside. Since the pressure will be always above ambient value, and the flow rate is low, cavitation would not be expected during the pressure and flow loading. The media used in the circuit would be incubated at 5% CO2, 37 °C for 30 minutes to establish the correct pH.
5.5.2. Mechanical loading system

The pressure-loading loop was successfully integrated with the fluid shear loading loop. The maximum pressure was chosen to be 100 PSI (690 kPa) based on experience during testing. This was achieved by using a pressure regulator cartridge with lower opening pressure. Even though this pressure level is lower than the original design, it is still an order of magnitude larger than the highest pressure applied to osteocytes in previous study (Liu, Zhao et al. 2010); and at the same order of magnitude as the calculated pressure at the lacunar-canalicular porosity (Zhang, Weinbaum et al. 1998). By using a lower pressure than the maximum of the system, we have increased reliability by reducing seal failure and leakage. Another consideration being at this pressure level, a prototype made from polycarbonate could be used instead of metal (stainless steel). The transparency of polycarbonate afford visibility of the loading space during flow. This is beneficial in de-gassing the system.

5.5.3. Mechanical loading chamber for osteocytes in vitro

The design of the cell chamber has been finalized and tested by applying pressure shear stress to MLO-Y4 cells. The percentage of apoptotic cells was reduced in both loading conditions, confirming previous studies (Bakker, Klein-Nulend et al. 2004, Liu, Zhao et al. 2010). When both loading types were applied at the same time, the reduction
in apoptosis was doubled, from 3-fold to 6-fold. Based on this additive effect, it hinted at independent pathways triggered by these two types of mechanical loading.

5.6. Limitations

5.6.1. Loading regime relevance to *in vivo* environment

The loading system developed in this study was able to apply physiological level of concurrent shear stress and hydraulic pressure to cells seeded on glass slides. The maximum hydraulic pressure that could be safely produced by the system was 600 kPa, which was still much less than the maximum physiological pressure of 5 MPa (Gardinier, Townend et al. 2010). The magnitude of the mechanical loading types could be controlled with an accuracy of within 10%, which was performed by the pressure regulator. However the frequency component of the loads could not be readily changed within the current system. It was set at 1 Hz for the duration of the experiments. Though this frequency of loading was within the physiological range, continuous loading of the same frequency is rare in nature. A related parameter, the phase difference of the fluid shear and hydraulic pressure could not be accurately controlled. In contrast, the *in vivo* shear and pressure were always nearly 180 degrees out of phase due to their coupled nature (Goulet, Hamilton et al. 2008).

5.6.2. Osteocyte model
The *in vitro* nature of this system should also be taken into consideration when evaluating any data produced by this system. The osteocyte model used in the study, MLO-Y4 cells, is different from native osteocytes in terms of gene expression and released signalling molecules (Yang, Harris et al. 2009). However, due to its widespread usage and extensive body of knowledge regarding this cell line, it was used as a standard for studying osteocytes *in vitro*. Furthermore, the mechanotransduction pathways in MLO-Y4 cells had been shown to be largely intact. Therefore, the experiments were designed with controls that the mechanically loaded groups could be compared against.

5.6.3. Culturing environment

The culture environment of cells could also lead to changed cell response to mechanical stimuli. The cells were cultured on glass slides for the mechanical loading experiments. The elastic modulus of soda lime glass (material the glass slide is made off) is actually similar to that of hydroxyapatite, the calcified matrix in bone (Reilly and Burstein 1975). However the glass substrate is missing many of the non-collagenous proteins that are distributed in bone. Therefore, the experiments were performed such that all of the samples, including the controls, had the same culturing conditions at all times.

5.7. Future work
To improve the maximum pressure of the system, the cell chamber needs to be made from material with higher elastic modulus. A good candidate is stainless steel has elastic modulus that is 100 fold higher than the plastic materials used in this study. In addition, stainless steel is biocompatible and has already been used in implants in clinical settings. It could be sterilized by pressurized steam. The design of the chambers do not need to be changed when adapting to stainless steel. The drawback are the material and machining costs; both are higher than plastics used here.
Chapter 6

6. Effect of hyperglycemia on osteocyte sensitivity to mechanical loading *in vitro*

6.1. Introduction

It is well accepted that hyperglycemia in diabetic patients adversely affects osteoblast function, resulting in retarded bone accumulation, bone loss, and an elevated risk of bone fracture (Rees and Alcolado 2005, Blakytny, Spraul et al. 2011, McCabe, Zhang et al. 2011). However, the effects of hyperglycemia on the most abundant bone cells, osteocytes, are not fully understood. Distributed in the bone matrix and well-connected with each other, as well as with cells lining the bone surface, osteocytes serve not only as primary sensors that detect and respond to mechanical stimulation, one of the most potent anabolic signals, but also as a paracrine regulator of osteoblasts and osteoclasts via the sclerostin/SOST and OPG/RANKL pathways (Bonewald 2007, Fritton and Weinbaum 2009). Whether hyperglycemia would alter osteocyte mechanosensitivity and whether the alterations are involved in the skeletal pathology associated with diabetes have not been elucidated.
In this study, we have examined secretion of anabolic cytokine, PGE2, catabolic cytokine RANKL and osteocyte apoptosis, which play a role in initiation of bone remodelling.

6.2. Hypothesis

We hypothesize that hyperglycemia suppress the effects of mechanical loading (oscillatory fluid shear) on osteocytes in terms of the prevention of apoptosis, the release of PGE2, and the reduction in RANKL secretion.

6.3. Methods

6.3.1. Cell Culture

MLO-Y4 osteocyte-like cells (gift from Dr. Lynda Bonewald, UMKC) were maintained in alpha-MEM with 2.5% fetal bovine serum, 2.5% calf serum, and 1% penicillin/streptomycin (Invitrogen).

MLO-Y4 cells were seeded on rat tail type I collagen (BD Biosciences) coated glass slides (75 x 38 mm) at a density of 3000 cells/cm2 and incubated for 72 hours in each of the following media: i) Normglycemic control: regular alpha-MEM culture medium containing 5.5mM glucose, ii) osmotic control: regular medium supplemented
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with 20 mM of D-mannitol (Sigma); and iii) hyperglycemic: regular medium supplemented with 20 mM D-glucose (Sigma).

6.3.2. Fluid shear stress loading

Parallel plate flow chambers were used to apply 1 Pa of oscillating shear stress at 1Hz for 2 hours. During the assembly of the flow chambers, fresh media of each type were used to fill the flow system. Then, the cells were incubated at 37 °C with fresh media. Media were collected after 24 hours of incubation.

6.3.3. Cell lysis

After 24 hours of incubation post mechanical loading, the cells were lysed with lysis buffer (CellSignal) according to manufacture instructions. The cells were washed with PBS to remove residue media. 400 μL of lysis buffer were added to the top of the slides. The slides were then incubated on ice for 5 minutes. The mixture was scraped off the slides with cell scrapers, and transferred to centrifuge tubes. The samples were sonicated; then centrifuged at 14,000 g for 10 minutes at 4 °C. Supernatant was removed from each sample to be used in apoptosis analysis.

6.3.4. Protein Quantification
A sandwich ELISA (R&D Systems) was used to quantify OPG and RANKL levels in conditioned media. PGE2 levels were quantified using a metabolite based EIA kit (Cayman). Total protein concentration in the cell lysate of each sample was determined using a colorimetric kit (Pierce).

6.3.5. Apoptosis Measurement

A fluorescent DEVD based caspase 3/7 kit (Promega) was used to quantify caspase levels in MLO-Y4 cells according to manufacture instructions. The DEVD molecules would irreversibly bind to caspase 3 and 7 in the homogenized mixture obtained from the cell lysis step. The DEVD only become fluorescent when bound to caspase3/7. For each sample 100 μL of the lysate was mixed with 100 μL of the DEVD solution in one well of a 96 well plate. The samples were incubated for 1 hour at the room temperature. Then a standard plate reader was used to measure the fluorescence, which had the excitation of 499 nm and emission of 521 nm.

6.3.6. Statistical analysis

ANOVA and post-hoc Tukey test were used to determine the significance of the variations between experimental groups. P < 0.05 was defined as significant.
6.4. Results

Two categories of independent variables were used in this study: mechanical loading (in the form of fluid shear stress) and media type. Since the goal was to investigate the impact of addition of glucose in media, the media type was used as the main independent variable in the results. For each type of media, the measured response from the mechanically loaded group was normalized to that of the static group. Therefore a value of 1 indicated the measured outcome did not vary with the fluid shear stress stimulation. A value of < 1 indicated the measured response was reduced by fluid shear stress, and vice versa for > 1.

Osteocytes in the normglycemic or mannitol-supplemented media showed decreased apoptosis induced by fluid flow (Figure 6-1A), which was abolished in hyperglycemic medium. In normglycemic and osmotic control media, fluid flow resulted in decreased secretion of RANKL by osteocytes, which was inhibited in hyperglycemic medium (Figure 6-1B). PGE2 production increased in fluid flow conditions in normglycemic and osmotic control media was suppressed in hyperglycemia media (Figure 6-1C).
6.5. Discussion

This study investigated how hyperglycemia affected protein expression of markers involved in bone remodelling in osteocytes under mechanical loading. Hyperglycemia abolished mechanical loading induced changes in RANKL and PGE2 levels released by MLO-Y4 cells. Cells in osmotic control condition did not exhibit the same effect, suggesting hyperglycemic condition may have direct effects on osteocytes, adversely influencing osteocyte’s ability to respond to mechanical loading. This effect may be responsible for the impaired bone structure and properties seen in diabetic patients.

6.6. Limitations
Osteocyte response to mechanical loading is very dynamic, ranging from with seconds to days, and even weeks (Duncan and Turner 1995, Klein-Nulend, Bakker et al. 2013). The mechanical loading experiments from this study were done at a single time point, which was 72 hours after the cells had been exposed to hyperglycemic media. Then the cells were incubated for 24 hours after the mechanical loading. Since the loading experiment was 2 hours, the total period of time that the MLO-Y4 cells were cultured in hyperglycemic media was 96 hours.

In this *in vitro* study, the concentration of D-glucose was maintained at 25.5 mM. However, *in vivo*, the glucose concentration varies with time as well ingestion of food for diabetic patients (Monnier, Mas et al. 2006). From the same study, it was shown that acute glucose swings could induce more oxidative stress compared to constantly elevated glucose level.

6.7. Future work

A time point study with higher resolution could elucidate the temporal dynamic of osteocyte signalling under a hyperglycemic environment.

Since oxidative stress had been implicated as one of the primary culprits of cell dysfunction in diabetic patients (Monnier, Mas et al. 2006), using different profiles of glucose concentration could better approximate the *in vivo* condition.
Chapter 7

7. Discussion

The goal of this thesis was to investigate the release of bone remodelling signals by osteocytes in response to mechanical loading in stressed conditions. Physical damage, and high glucose concentration were used to simulate physical and chemical stresses that the osteocytes would experience. Bone develops microdamage under physiological loading due to its hard and brittle nature in term of material properties in comparison with the surrounding tissues. The microdamage in bone consists of linear cracks, which have micron sized displacements, and diffuse damage, which is material faults at least one order of magnitude less in displacement (Schaffler, Pitchford et al. 1994). Osteocytes have been shown to be affected by the linear cracks from microdamage (Hazenberg, Freeley et al. 2006). These cracks cause cellular deformation to the point of breaking the cell membrane. Cell death has been observed in osteocytes damaged in these cracks. Apoptosis of the surrounding osteocytes has also been observed (Clark, Smith et al. 2005). Bone remodelling by the basic multicellular unit repairs the linear cracks only when both osteocytes and mechanical loading are present at the damage site (Waldorff, Christenson et al. 2010). Without this targeted remodelling, micro-cracks would accumulate and lead to decreased bone strength as well as higher risk of fracture (Burr, Forwood et al. 1997). Therefore, the first aim of this thesis investigates the role of
mechanical loading in osteocyte signalling related to bone remodelling. The findings were presented in Chapter 4. It was found that mechanically loading had an amplifying or additive effect in the release of remodelling signals.

The pathology of diabetes mellitus results in reduced bone strength, sometime even in the absence of reduced mineral density (McCabe, Zhang et al. 2011). This led to the hypothesis that a dysregulation of the remodelling process is the reason behind this phenomenon. At the cellular level, osteocyte response to mechanical loading may be directly affected by the higher-than-normal glucose level this is present in this condition. Indeed, this was the finding presented in Chapter 6. Briefly, high glucose concentration abolished the anti-apoptotic effect of mechanical loading in osteocytes. The up-regulation of PGE2 and down-regulation of RANKL by mechanical loading was also abolished. It could be argued that the inhibition of these remodelling signals could cause accumulation of micro-cracks in bone and lead to poor mechanical strength. The work presented in this thesis aims to demonstrate the interactive effect of cellular stress form these two types of stimuli on osteocyte release of signalling molecules in response to mechanical loading.

In vivo, osteocytes experience both hydraulic pressure and fluid flow shear stress. In fact, these are coupled forces in the bone: loading increases the fluid pressure, which is relieved by the flow of the interstitial fluid along the pressure gradient. The lacuna-canalicular space has the smallest porosity in the bone, relative to the vascular space (Cowin, Gailani et al. 2009). The pressure gradient would drive fluid flow out of the lacuna-canalicular space under loading and vice versa during unloading. Previous studies had been focused on a single mode of mechanical loading, either pressure (Liu, Zhao et
al. 2010) or shear stress (Tan, Kuijpers-Jagtman et al. 2006, Ponik, Triplett et al. 2007, Wang, Wan et al. 2007, Riddle and Donahue 2009, Huesa, Helfrich et al. 2010). Both had been shown to be significant stimuli to the osteocytes. The question naturally follows is how osteocyte respond to both types of loading which is the case in vivo. The mechanical loading system presented in Chapter 5 aims to answer this question. The loading system was validated with finite element analysis and empirical testing. A high-throughput prototype was used to investigate the response of osteocytes to concurrent pressure and shear loading. Osteocyte apoptosis was reduced in concurrent loading compared with single mode loading. Concurrent loading also altered osteocyte morphology similar to shear only. Interestingly, only concurrent loading induced actin-rich filopodia, which had been implicated as sensors (Heckman and Plummer III 2013). The mechanical loading system presented here serves as a platform to study the effect of combinations of physiological levels of pressure and fluid shear on cells in vitro. This thesis presented the unique responses of osteocyte to the concurrent loading. Since pressure-drive flow is common in vivo, this system could also be used to study the response of other cell types, such as chondrocyte, endothelial and mesenchyme cells.

7.1. **In vitro model to study the cellular response to physical damage**

An in vitro system was developed to create sub-cellular physical damage that is similar to what osteocytes experience around microdamage in bone in vivo. This was
achieved with an array of needles with micro sized tips. A mechanical platform was designed and made to allow for precise adjustment of the height of the array of these needles. The design and validation of the system is presented in Chapter 4. The osteocyte culture showed similar cell viability distribution and expression of VEGF to \textit{in vivo} experiments (Kennedy, Herman et al. 2012) after they were physically damaged by this system (Figure 4-3, Figure 4-7). Using this system, the early remodelling signals released by osteocytes were elucidated. The media concentration of VEGF and PGE2 increased 4-fold in the group with both mechanical loading and physical damage. Where as each of the stimulus only increased these signals by 2-fold.

7.2. \textit{In vitro} model to study osteocyte mechanotransduction

Osteocyte signals that control bone remodelling change in response to changes in their mechanical environment. At the length scale of cells, mechanical loading on the bone imparts pressure to the bone matrix. Subsequently this pressure drives fluid in the bone pores to flow toward the blood vessel, which acts as a sink that is analogous to the ground in electrical circuits. However, in the past, only one of these coupled forces has been used to study osteocyte mechanotransduction. In Chapter 5 of this thesis, a system that is able to apply both fluid flow shear stress and hydraulic pressure was developed to better simulate the forces that osteocytes would have experience \textit{in vivo}. The loading circuit was validated using SimuLink in MatLab (Figure 5-2 C). The integrity of the cell
loading chambers under maximum load was validated using finite element analysis (Figure 5-3).

7.3. Effect of hyperglycemia on osteocyte mechanotransduction

In Chapter 6 of this thesis, I present the investigation on how high glucose level affect osteocyte mechanotransduction. Hyperglycemia abolished mechanical loading induced changes in RANKL and PGE2 levels released by MLO-Y4 cells while osmotic control medium having the same elevated osmolarity did not have significant effects (Figure 2-1). Osmotic controls (mannitol treated groups) did not exhibit the same adverse effect on osteocytes, suggesting that the observed suppression in hyperglycemic condition was not a result of elevated osmolality. Instead, elevated glucose levels in diabetic patients may have direct effects on osteocytes, adversely influencing osteocyte’s ability to respond to mechanical loading.
Chapter 8

8. Conclusions and future work

8.1. Conclusions

The work presented in this thesis involves the investigation of how osteocyte mechanotransduction was affected by physical cellular damage and high glucose concentration. A new mechanical system developed to apply sub-cellular damage that is similar to what osteocyte experience in bone micro-cracks. Another system was developed in an effort to apply concurrent oscillatory fluid shear and pressure to cells in vitro. It was found that:

1. Sub-cellular damage of osteocyte in vitro produced similar spatial distribution of viable cells and expression of VEGF within the cells.

2. Osteocytes had higher response of PGE2 and VEGF release to mechanical loading 24 hours after physical cellular damage.

3. Osteoclast differentiation was reduced by osteocytes which had been subjected to combinations of physical damage and mechanical loading.
4. High glucose concentration abolished the ability of osteocyte to respond to mechanical loading in terms of apoptosis, PGE2 and RANKL release.

5. The new loading system was able to supply concurrent oscillatory fluid shear and pressure force to cells that were cultured on glass slides. The system was further validated by demonstrating decreased osteocyte apoptosis after combinations of fluid shear and pressure.

8.2. Future work

In Chapter 4, it was found that after osteocytes had been subjected to physical damage, their media suppressed osteoclast differentiation (Figure 4-8). This was a surprising result, since bone micro-cracks was expected to trigger osteoclast formation as a part of the remodelling process. However, based on the time point of the study (24 hour after damage), it is likely too early for the osteocytes to support osteoclast differentiation. Indeed, the osteoclast differentiation signal RANKL was only up-regulated 3 days (Kennedy, Herman et al. 2012) to 7 days (Mulcahy, Taylor et al. 2011) after micro-crack formation. In Chapter 4, RANKL and IL-6 were shown to be not affected by physical cellular damage. Therefore some other signals were responsible for causing the inhibitory effect. Osteocytes signals that initiate remodelling have a dynamic temporal expression profile. New microscopy techniques of deep within bone tissues (Dong, Haupert et al. 2014) enable the observation of these signals with appropriate gene modifications in vivo. Understanding of the spatial and temporal regulation of bone remodelling would allow
optimization of therapeutic agents for the treatment of bone pathologies such as osteoporosis.

Chapter 6 showed that high glucose concentration abolished osteocyte response to mechanical loading in terms of remodelling signals and apoptosis (Figure 6-1). The implication is that the bone would have retarded mechanically induced remodelling in hyperglycemic conditions such as the case for diabetic patients. Poor bone strength could result from this deficiency due to accumulation of microdamage (Burr, Forwood et al. 1997) or non-optimal distribution of bone material in relation to stress concentration (Cowin 1984). At the cellular level, it is unclear what mechanosensitive structures are affected by the elevated glucose concentration. Strain activated ion channels could be affected due to the change in membrane fluidity, which is controlled partially by glycosylation of membrane proteins (Mazzanti, Rabini et al. 1997). The biochemistry of the cell could be indirectly affected by the build-up of oxidative products (Monnier, Mas et al. 2006). Insulin like growth factor 1 (IGF-1) signalling could play a role in osteocyte mechanotransduction in high glucose concentration (Sheng, Zhou et al. 2013). Recently, mechanical loading is shown to activate insulin like growth factor 1 receptor (IGF1R) through integrins in osteoblasts (Tahimic, Long et al. 2016). Since osteogenic cells also express IGF-1 (Kronenberg 2003), autocrine signalling may play a role in the mechanosensitivity of IGF-1 to IGF1R signalling. In future studies, the expression and response of IGF-1 and IGF1R in osteocytes should be elucidated in the context of mechanotransduction. Understanding of the mechanobiology of hyperglycemia-induced bone pathology could open up a new avenue of treatment based on mechanical stimulation in addition to pharmaceutical means.
Currently, the measure of osteocyte response to mechanical loading had been through apoptosis and release signalling molecules. The intracellular signal transduction pathway in osteocytes was only beginning to be studied recently. Currently the Wnt signalling pathway has shown to be mechanically triggered (Bonewald and Johnson 2008). Since the action of the Wnt pathway is very specific. Other signal transduction avenues could be possible. One candidate is the Rho kinase (ROCK), which is a downstream target of GTP-binding protein Rho. ROCK regulate actin cytoskeleton, through which, it controls cell growth, migration, metabolism and apoptosis (Riento and Ridley 2003). ROCK has already been shown to be active in osteoblastic cells (Yoshida, Clark et al. 2009). Based on these evidence, ROCK is likely active in osteocytes and a possible mechanosensory pathway. Using MLO-Y4 cells as the model for osteocytes, they were subjected to fluid shear stress and a ROCK inhibitor (10 μM Y27632). Preliminary experiment has shown that ROCK inhibition abolished the loading-induced reduction of apoptosis (Appendix A-2). Signal transduction by kinases such as ROCK requires molecule-molecule contact. Recent development of super-resolution microscopy (Wang, Moffitt et al. 2014) could be utilized to image these events to uncover the signal transduction cascade induced by mechanical loading. This will add to the knowledge of how cells respond to mechanical loading, one of the fundamental forces in nature.
References


Appendix

Appendix A-1: Time point study of osteocyte genes after physical damage

![Graph of RANKL/OPG](image1.png)

![Graph of RANKL/18s](image2.png)
Figure A-1. Time point measurement of RANKL/OPG ration, RANKL, OPG, and IL-6 mRNA, normalized to 18s rRNA.
Appendix A-2: percentage of apoptotic osteocytes after treatment of unloading and ROCK inhibitor Y27632.

Figure A-2. The percentage of apoptotic osteocytes after treatment of combinations of unloading and Y27632. MLO-Y4 cells in all groups were subjected to fluid shear stress for 24 hours. Then the loading was stopped in the unloading groups for 6 hours. During this time, 10 μM of Y27632 was added to the two of the groups. Then apoptosis was measured using the Apop percentage assay.